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A novel serine kinase activated by rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and STE20

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We identified three proteins in neutrophil cytosol of molecular size 65, 62 and 68 kDa which interact in a GTP-dependent manner with rac1 and CDC42Hs, but not with rho. Purification of p65 and subsequent peptide sequencing revealed identity to rat brain PAK65 and to yeast STE20 kinase domains. Based on these sequences we screened a human placenta library and cloned the full-length cDNA. The complete amino acid sequence of the human cDNA shares ~73% identity with rat brain PAK65; within the kinase domain the human protein shares >95% and ~63% identity with rat PAK65 and yeast STE20 respectively. The new human (h)PAK65 mRNA is ubiquitously expressed and hPAK65 protein is distinct from either human or rat brain PAK65. Recombinant hPAK65 exhibits identical specificity to the endogenous p65; both can bind rac1 and CDC42Hs in a GTP-dependent manner. The GTP-bound forms of rac1 and CDC42Hs induce autophosphorylation of hPAK65 on serine residues only. hPAK65 activated by either rac1 or CDC42Hs is phosphorylated on the same sites. Induction of hPAK65 autophosphorylation by rac1 or CDC42Hs stimulates hPAK65 kinase activity towards myelin basic protein and once hPAK65 is activated, rac1 or CDC42Hs are no longer required to keep it active. The affinities of rac/CDC42Hs for the non-phosphorylated and phosphorylated hPAK65 were similar. hPAK65 had only a marginal effect on the intrinsic GTPase activity of CDC42Hs, but significantly affected the binding and GAP activity of p190. These data are consistent with a model in which hPAK65 functions as an effector molecule for rac1 and CDC42Hs.

Key words: GTPase targets/MAP kinase/neutrophil kinase

Introduction

The rho-like proteins, like other GTPases, cycle between the active GTP-bound form and the inactive GDP-bound state (Nobes and Hall, 1994). Regulation of these forms has been shown to be controlled by several proteins, including guanine nucleotide exchange factors (GEF) such as Dbl (Hart *et al.* 1991) and GTPase activating proteins such as p190 (Settleman *et al.*, 1992; Boguski and McCormick, 1993). Members of the rho family of proteins, including RhoA, B and C, rac1 and 2, CDC42Hs and TC10, share ≥50% sequence identity with each other and 30% identity with other ras-like proteins (Nobes and Hall, 1994). Insight into the physiological function of rho and rac proteins has emerged from recent reports (Ridley and Hall, 1992; Ridley *et al.*, 1992), in which rapid cytoskeletal effects were detected when rho and rac proteins were microinjected into Swiss 3T3 fibroblasts. Activated rho induces stress fiber formation and focal contact (Ridley and Hall, 1992), whereas activated rac induces the formation of membrane ruffles and lamelipodia (Ridley *et al.*, 1992). rho proteins are also implicated in other physiological roles associated with cytoskeletal rearrangements, such as cell motility (Takaishi *et al.*, 1993), cytokinesis (Kishi *et al.*, 1993) and lymphocyte aggregation (Tominaga *et al.*, 1993).

While the physiological function of CDC42 was shown to be essential in bud formation in yeast (Johnson and Pringle, 1990), no similar physiological function has been described for its mammalian homolog, CDC42Hs. However, a hint for its role in mammalian cells came from a study demonstrating that the proto-oncogene Dbl exhibits GEF activity on rho and CDC42Hs (Hart *et al.*, 1991). This observation suggests a role for rho-like proteins in cell transformation. However, not all proteins containing the Dbl domain demonstrate nucleotide exchange activity on rho-like proteins. For example, vav (Gulbins *et al.*, 1993), Ect2 (Miki *et al.*, 1993), ras GRF and bcr (Boguski and McCormick, 1993) do not exert nucleotide exchange activity on rho and the Dbl domains of bcr and ras GRF do not transform cells. A recent study suggests a direct link between rho and Dbl *in vivo* by demonstrating that vav and Dbl transformation is mediated by rho (Khosravi-Far *et al.*, 1994).

From molecular function studies in phagocytes it was demonstrated that rac 1 and 2 are involved in the control of superoxide generation by NADPH oxidase (Abo *et al.*, 1991; Knaus *et al.*, 1991). Activated rac, together with two other oxidase cytosolic components, p47-*phox* and p67-*phox*, assemble with membrane-bound cytochrome b_{558} to form an active oxidase (Segal and Abo, 1993). More recently, it was shown that the effector molecule for rac in this system is p67-*phox*. (Diekmann *et al.*, 1994).

An additional molecular effector for rac and CDC42Hs was shown to be a rat brain serine/threonine kinase, PAK65 which is activated by rac1 and CDC42Hs (Manser *et al.*, 1994). Other studies have suggested that CDC42Hs and rho activate PI3 kinase (Zhang *et al.*, 1993; Zheng *et al.*, 1994).

In the following report we identify three novel effector proteins for rac1 and CDC42Hs in neutrophils. We have purified one of them, cloned its cDNA, expressed the recombinant protein and characterized its biochemical properties.

Results

Identification of effector proteins in neutrophils for rac1 and CDC42Hs

The overlay assay for GTPases was initially described as a method to detect GTPase activating proteins (GAPs) (Manser *et al.*, 1992) for rho-like proteins. By probing a nitrocellulose filter containing lysate with [γ-^{32}P]GTP-bound rho-like proteins it is possible to detect proteins on the filter which can affect the GTP hydrolysis rate. Since GAPs catalyze the release of the γ-^{32}P from GTP, the loss of radiolabeled ^{32}P from the GTPase probe can be visualized as clear bands over a dark background. However, by monitoring the probing conditions it is also possible to detect effector proteins which inhibit the release of ^{32}P and thus can be visualized as dark bands. Using this method we were able to detect three major bands clustered near the 68 kDa marker in neutrophil cytosol. Fractionation of neutrophil cytosol on a Mono Q column resolved these into three distinct bands of molecular size 62, 65 and 68 kDa, which were detected only when the filter was probed with the GTP-bound form of rac1 and CDC42Hs and not with GTP−rho (Figures 1 and 3).

Isolation of hPAK65 cDNA clone

By several conventional purification steps we were able to isolate the p65 band, which was the most abundant of the three proteins. Digestion of p65 and subsequent amino acid analysis yielded the following amino acid sequence: STMVGTPYWMAPEVVTR, which is closely related to a sequence within the serine/threonine kinase domain of yeast STE20 (Ramer and Davis, 1993) and 100% identical to a rat brain serine/threonine kinase, PAK65 (Manser *et al.*, 1994). Based on this sequence and the previously published sequence of rat brain PAK65 (Manser *et al.*, 1994), primers were designed to amplify PAK cDNA from several commercially available human cDNA libraries. Although the expected product was detected in several tissue-specific libraries, relatively large amounts were detected in a human placenta library. The generated PCR product was used to screen and clone a full-length cDNA.

Amino acid homology

The nucleotide and amino acid sequence of the new human PAK65 clone, which will be referred to here as hPAK65, is shown in Figure 2. The complete amino acid sequence of hPAK65 shares ~73% identity with the previously isolated PAK65 from rat brain (Manser *et al.*, 1994) (Figure 2A) and shares >95% identity within the kinase domain (amino acids 230–507) (Figure 2B). In addition, hPAK65 exhibits ~63% identity with the kinase domain of STE20 (positions 620–876; Ramer and Davis, 1993) (Figure 2C). As reported for rat PAK65 (Manser *et al.*, 1994), the rac and CDC42Hs binding domains (amino acids 47–113) of hPAK65 also share some similarities with the rat brain PAK65 and STE20 regulatory domain.

Tissue distribution of hPAK65

We examined several human tissues and cell lines for the expression of hPAK65 mRNA by Northern and Western blot analysis. We generated a probe derived from the highly conserved kinase domain among STE20, rat brain

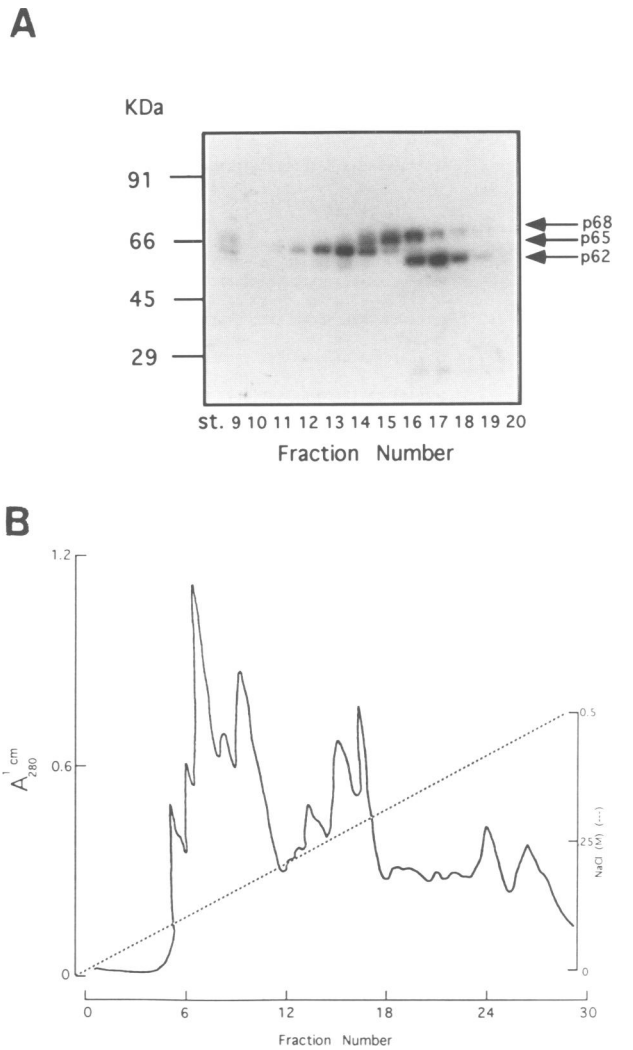

Fig. 1. Fractionation of neutrophil cytosol on a Mono Q column. (**B**) Neutrophil cytosol (10 ml, 10 mg/ml) was applied and eluted with a 30 ml salt gradient. (**A**) Collected fractions analyzed by the overlay assay were probed with [γ-^{32}P]GTP−CDC42Hs as described in detail in Materials and methods.

PAK65 and hPAK65. This allowed us to look at the expression of closely related messages. hPAK65-related mRNAs are ubiquitously distributed, with higher levels in skeletal muscle, ovary, thymus and spleen and 2- to 3-fold higher in the HL-60 cell line (Figure 3). Four RNA species were detected in most of the tissues, with sizes of ~7.5, 5, 4.4 and 3 kb. The 7.5 kb message was the predominant species in all the tissues except skeletal muscle, where the 7.5 and 3 kb mRNAs were roughly equal. In contrast, the cell lines HL-60, Molt-4, Raji and SW480 have equal amounts of all four species, whereas in brain a different sized mRNA was detected at ~3.3 kb. Based on results of genomic Southern blotting (results not shown), these multiple mRNA are most likely alternatively spliced forms of a single gene (Figure 3). In addition, we have analyzed PAK protein distribution by probing Western blots with antibodies raised against recombinant hPAK65 or the N-terminal peptide of rat brain PAK65. A brain-specific PAK protein was detected in both rat and human with the rat brain antibody. Partially purified p65 and recombinant hPAK65 did not cross-react with this

A

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AGGGGAGGACACACTTCTGGCAAACGTTTCTCAAATCTGCTTCATCCAATGTGAAGTTCATCTTGCAGCATTTACTATGCACAACAGAGTAACTATCGGGTCCTGTGGACAGCTCACCTA 120
GTGGCAATGGCTCCAGGCTCCCGGACATCCCGTCTCCTGGCTTTTGCCTGCTCTGCCCTGCCCTGGCTTCAAGAGGCTGGTGCGTCCAAACCGTTCCGTTATCCAGGCTTTTTGACCACGC 240
TATGCTCCAAGCCCATGCCGCGCACCAGCTGGCCATTGACACCTACCAGGAGTTTGAAGAAACCTATATCCCAAAGGACCAGAAGTATTCATTCCTGCATGACTCCCAGACCTCCTTCTGC 360
TTCTCAGACTCTATTCCGACACCCTCCAACATGGAGGAAACGCAACAGAAATCCAATCTAGAGCTGCTGTCAGCCAATCACAGTTTGAAACCTTTGCCCTCTGTTCCAGAAGAGAAAAAG 480
                                M  E  E  T  Q  Q  K  S  N  L  E  L  L  S  A  N  H  S  L  K  P  L  P  S  V  P  E  E  K  K
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 P  R  H  K  I  I  S  I  F  S  G  T  E  K  G  S  K  K  K  E  K  E  R  P  E  I  S  P  P  S  D  F  E  H  T  I  H  V  G  F
GATACTGTTACTGGAGAATTCACTGGCATGCCAGAACAGTGGGCTCGATTACTACAGACCTCCAATATCACCAAACTAGAGCAAAAGAAGAATCCTCAGGCTGTGCTGGATGTCCTAAAG 720
 D  T  V  T  G  E  F  T  G  M  P  E  Q  W  A  R  L  L  Q  T  S  N  I  T  K  L  E  Q  K  K  N  P  Q  A  V  L  D  V  L  K
TTCTACGACTCCAACACAGTGAAGCAGAAATATCTGAGCTTTACTCCTCCTGAGAAAGATGGCTTTCCTTCTGGAACACCAGCACTGAATGCCAAGGGAACAGAAGCACCCGCAGTAGTG 840
 F  Y  D  S  N  T  V  K  Q  K  Y  L  S  F  T  P  P  E  K  D  G  F  P  S  G  T  P  A  L  N  A  K  G  T  E  A  P  A  V  V
ACAGAGGAGGAGGATGATGATGAAGAGACTGCTCCTCCCGTTATTGCCCCGCGACCGGATCATACAAAATCAATTTACACACGGTCTGTAATTGACCCTGTTCCTGCACCAGTTGGTGAT 960
 T  E  E  E  D  D  D  E  E  T  A  P  P  V  I  A  P  R  P  D  H  T  K  S  I  Y  T  R  S  V  I  D  P  V  P  A  P  V  G  D
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 S  H  V  D  G  A  A  K  S  L  D  K  Q  K  K  K  T  K  M  T  D  E  E  I  M  E  K  L  R  T  I  V  S  I  G  D  P  K  K  K
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 Y  T  R  Y  E  K  I  G  Q  G  A  S  G  T  V  F  T  A  T  D  V  A  L  G  Q  E  V  A  I  K  Q  I  N  L  Q  K  Q  P  K  K
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 G  S  A  K  E  L  L  Q  H  P  F  L  K  L  A  K  P  L  S  S  L  T  P  L  I  M  A  A  K  E  A  M  K  S  N  R  .
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B

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rat p65 PAK  M S N N G L D V Q D K P P A P P M R N T S T M I G A G S K D P G T L N H G S K P L P P N P E E K K K  50
human p65 PAK M E - - - - E T Q Q K S N L E L L S A - - - - - - - - - - - - - - - N H S L K P L P S V P E E K K P  31

rat p65 PAK  K D R F Y R S I L A G D K T N K K K E K E R H E I S L P S D F E H T I H V G F D A C T G E F T G M P  100
human p65 PAK R H K I I S I F S G T E K G S K K K E K E R P E I S P P S D F E H T I H V G F D T V T G E F T G M P  81

rat p65 PAK  E Q W A R L L Q T S N I T K S E Q K K N P Q A V L D V L E F Y N S K K T S N S Q K Y M S F T D K S A  150
human p65 PAK E Q W A R L L Q T S N I T K L E Q K K N P Q A V L D V L K F Y D S N T V - - K Q K Y L S P T P P E K  129

rat p65 PAK  E D Y N S S N T L N V K T V S E T P A V P P V S E D E D D D D A T P P P V I A P R P E H T K S V Y  200
human p65 PAK D G F P S S G T P A L N A K G T E A P A V V - - - T E E E D D D E E T A P P V I A P R P D H T K S I Y  176

rat p65 PAK  T R S V I E P L P V T P T R D V A T S P I S P T E N N T T P P D A L T R N T E K Q K K K P K M S D E  250
human p65 PAK T R S V I D P V P A P V G - - - - - - - - - - - - - - - D S H V D G A A K S L D K Q K K K T K M T D E  212

rat p65 PAK  E I L E K L R N I V S V G D P K K K Y T R F E K I G Q G A S G T V Y T A M D V A T G Q E V A I K Q M  300
human p65 PAK E I M E K L R T I V S I G D P K K K Y T R Y E K I G Q G A S G T V F T A T D V A L G Q E V A I K Q I  262

rat p65 PAK  N L Q Q Q P K K E L I I N E I L V M R E N K N P N I V N Y L D S Y L V G D E L W V V M E Y L A G G S  350
human p65 PAK N L Q K Q P K K E L I I N E I L V M K E L K N P N I V N F L D S Y L V G D E L F V V M E Y L A G R S  312

rat p65 PAK  L T D V V T E T C M D E G Q I A A V C R E C L Q A L E F L H S N Q V I H R D I K S D N I L L G M D G  400
human p65 PAK L T D V V T E T C M D E A Q I A A V C R E C L Q A L E F L H A N Q V I H R D I K S D N V L L G M E G  362

rat p65 PAK  S V K L T D F G F C A Q I T P E Q S K R S T M V G T P Y W M A P E V V T R K A Y G P K V D I W S L G  450
human p65 PAK S V K L T D F G F C A Q I T P E Q S K R S T M V G T P Y W M A P E V V T R K A Y G P K V D I W S L G  412

rat p65 PAK  I M A I E M I E G E P P Y L N E N P L R A L Y L I A T N G T P E L Q N P E K L S A I F R D F L N R C  500
human p65 PAK I M A I E M V E G E P P Y L N E N P L R A L Y L I A T N G T P E L Q N P E K L S P I F R D F L N R C  462

rat p65 PAK  L E M D V E K R G S A K E L L Q H Q F L K I A K P L S S L T P L I A A A K E A T K N N - H  544
human p65 PAK L E M D V E K R G S A K E L L Q H P F L K L A K P L S S L T P L I M A A K E A M K S N R  507
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C

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human p65 PAK  Y T R Y E K I G Q G A S G T V F T A T D V A L G Q E V A I K Q I N L Q K Q P K K E L I I N E I L V M  280
STE20          Y A N L V K I G Q G A S G G V Y T A Y E I G T N V S V A I K Q M N L E K Q P K K E L I I N E I L V M  669

human p65 PAK  K E L K N P N I V N F L D S Y L V G D E L F V V M E Y L A G R S L T D V V T E T C M D E A Q I A A V  330
STE20          K G S K H P N I V N F I D S Y V L K G D L W V I M E Y M E G G S L T D V V T H C I L T E G Q I G A V  719

human p65 PAK  C R E C L Q A L E F L H A N Q V I H R D I K S D N V L L G M E G S V K L T D F G F C A Q I T P E Q S  380
STE20          C R E T L S G L E F L H S K G V L H R D I K S D N I L L S M E G D I K L T D F G F C A Q I N E L N L  769

human p65 PAK  K R S T M V G T P Y W M A P E V V T R K A Y G P K V D I W S L G I M A I E M V E G E P P Y L N E N P  430
STE20          K R T T M V G T P Y W M A P E V V S R K E Y G P K V D I W S L G I M I I E M I E G E P P Y L N E T P  819

human p65 PAK  L R A L Y L I A T N G T P E L Q N P E K L S P I F R D F L N R C L E M D V E K R G S A K E L L Q H P  480
STE20          L R A L Y L I A T N G T P K L K E P E N L S S S L K K F L D W C L C V E P E D R A S A T E L L H D E  869

human p65 PAK  F L K - L A K                                                                                  486
STE20          Y I T E I A E                                                                                  876
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Fig. 2. Nucleotide and deduced amino acid sequence of hPAK65. hPAK65 cDNA was cloned from a human placenta library. (**A**) The peptide sequence obtained from p65 purification is underlined where matched in the full-length sequence. (**B**) Comparision between the amino acid sequences of hPAK65 and rat brain PAK65. (**C**) Comparision between the kinase domains of hPAK65 and STE20.

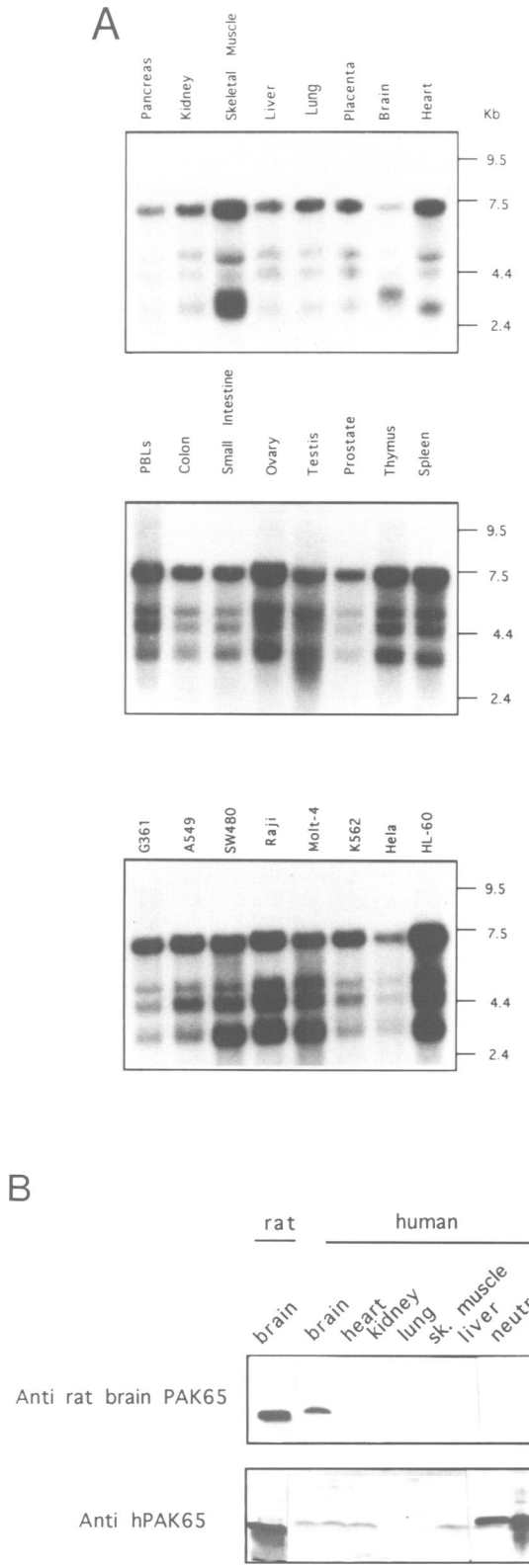

Fig. 3. Tissue distribution of PAK65. (**A**) A radioactively labeled probe generated by PCR from the kinase domain (nucleotide sequence 1009–1912) of hPAK65 cDNA was used to hybridize mRNA isolated from various tissues immobilized on Northen blots. Autoradiographs were exposed for 3 h. (**B**) Multiple human tissues and rat brain cytosol (75 μg/lane) were probed either with anti-rat brain antibody derived from the N-terminus of rat PAK65 or with anti-hPAK65.

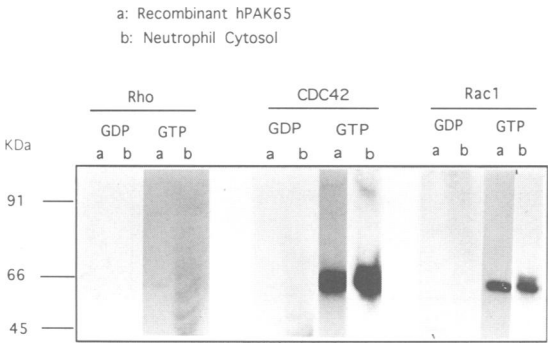

Fig. 4. hPAK65 binding specificity among rho-like proteins. Neutrophil cytosol (80 μg) or recombinant hPAK65 (2–3 μg) were applied to SDS−PAGE gels, blotted onto a PVDF filter and analyzed by the overlay assay. The filter was probed with the indicated GTPase pre-loaded with [γ-^{32}P]GTP or [β-^{32}P]GDP.

antibody. In addition, hPAK65 antibodies recognized PAK proteins in tissues other than brain, including heart, kidney and liver, and strongly reacted with partially purified neutrophil p65 (Figure 3B).

The binding specificity of hPAK 65

Recombinant hPAK65 or the endogenous PAK65 in neutrophil cytosol was detected only when the filter was probed with [γ-^{32}P]GTP−CDC42Hs and rac1, but not with rho A (Figure 4). No proteins were detected when the GTPase was preloaded with [β-^{32}P]GDP, consistent with the hypothesis that hPAK protein is an effector molecule for rac1 and CDC42Hs. The relative affinity as judged by the overlay assay is ~3- to 4-fold higher for CDC42Hs than for rac1 (Figure 4).

CDC42Hs and rac1 induce autophosphorylation of hPAK65

Incubation of hPAK65 with the activated form of rac1 or CDC42Hs in a kinase reaction containing [γ-^{32}P]ATP stimulated the autophosphorylation of hPAK65 (Figure 5A). No phosphorylation was observed with rho A or by omitting the GTPase and simply adding GTP (Figure 5A). Phosphorylation occurred in a dose-dependent manner only with the GTP or GTP-γS form of CDC42Hs or rac1. Maximal phosphorylation was obtained after 15 min at 30°C (data not shown). Phosphoamino acid analysis indicated that hPAK65 was phosphorylated on serine residues when activated by CDC42Hs and not on threonine or tyrosine residues (Figure 5B).

Since rac1 and CDC42Hs share ~72% sequence identity and apparently play different physiological roles, it is conceivable that rac1 and CDC42Hs may activate hPAK65 autophosphorylation on distinct sites. To test this hypothesis, hPAK65 was incubated with either rac1 or CDC42Hs in a kinase reaction with [γ-^{32}P]ATP and the phosphorylated protein was digested with three different enzymes: trypsin, chymotrypsin and Glu-C. This treatment resulted in the generation of radiolabeled phosphopeptides which were resolved on a 16% Tricine gel. Identical phosphopeptide profiles were generated from digestion of hPAK65 activated by either rac or CDC42Hs (Figure 5C). These results suggest that rac and CDC42Hs stimulate hPAK65 to autophosphorylate on the same sites.

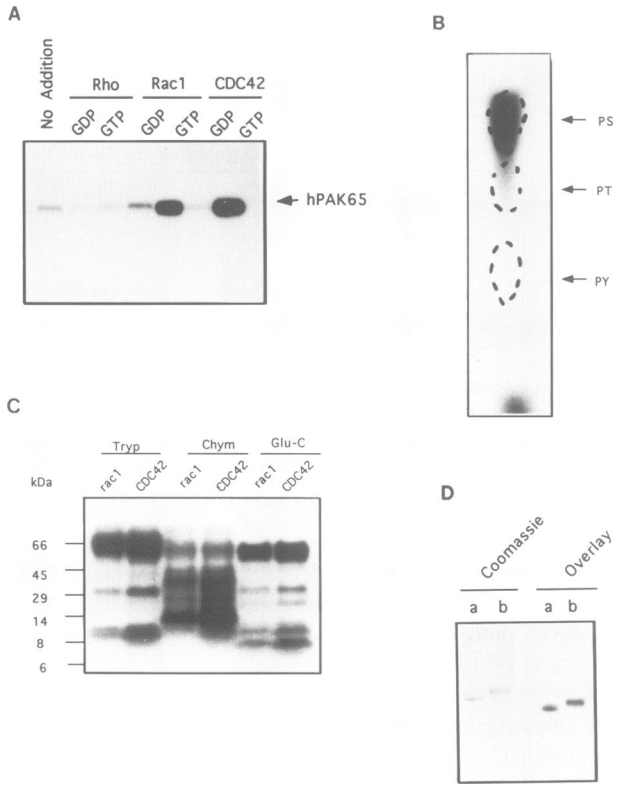

Fig. 5. Activation of hPAK65 autophosphorylation. (**A**) Recombinant hPAK65 (1–2 μg) immobilized on beads in 40 μl kinase buffer was incubated with 1–2 μg of the indicated GTPases which were pre-loaded with either GTP or GDP. The reaction was incubated for 20 min at 30°C with 50 μM ATP and 5 μCi [γ-^{32}P]ATP. Phosphorylated proteins were analyzed by SDS−PAGE followed by autoradiography. (**B**) Radiolabeled phosphorylated hPAK (4 μg), mediated by CDC42Hs, was hydrolyzed in 6 N HCl, 110°C for 2 h and the phosphoamino acids were separated by thin layer electrophoresis. ^{32}P-Labeled residues were detected by autoradiography. (**C**) Proteolytic digestion was performed with the indicated enzymes on hPAK65 which was either pre-incubated with rac1 or CDC42Hs in a kinase reaction containing [γ-^{32}P]ATP. The radiolabeled peptides were resolved on a 16% Tricine gel and were visualized by autoradiography. (**D**) Either unphosphorylated (a) or phosphorylated (b) hPAK65 (2 μg each), induced by CDC42Hs, were run in SDS−PAGE, stained with Coomassie Blue and tested for CDC42Hs binding by the overlay assay.

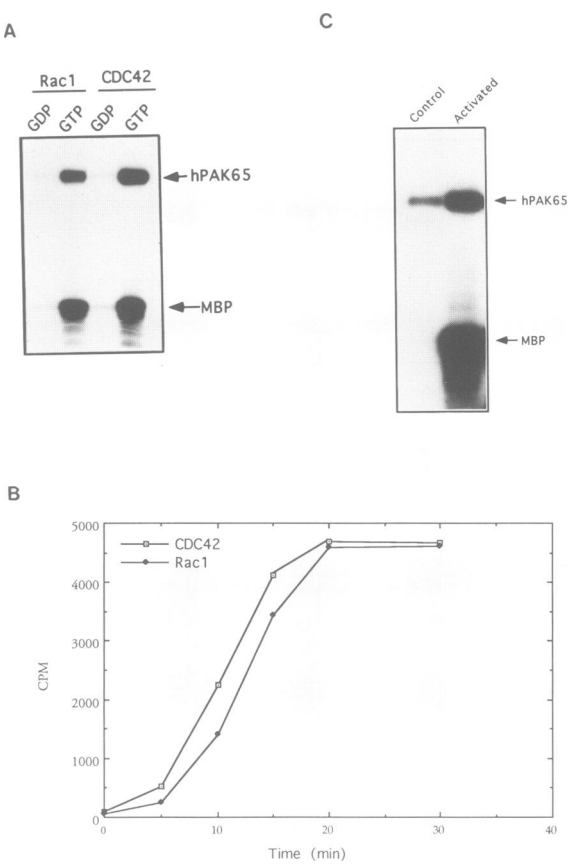

Fig. 6. Activation of hPAK65 kinase activity. (**A**) MBP (3 μg) was included with hPAK65 in a kinase reaction as described in the legend to Figure 5A. (**B**) Kinase reaction mixture (5 μl) was removed every 5 min and the reaction was stopped by adding SDS sample buffer. The phosphorylated MBP was separated by 14% SDS−PAGE, the band was excised and the incorporated ^{32}P was counted. (**C**) hPAK65 (4 μg, 30 μl beads) was first incubated for 20 min with 3 μg CDC42Hs in the presence (activated) or absence (control) of ATP in a kinase reaction. To remove CDC42Hs, the hPAK65 beads were washed three times and hPAK65 was subjected to a second kinase reaction containing [γ-^{32}P]ATP and MBP.

Comparison of the binding of CDC42Hs to phosphorylated versus unphosphorylated hPAK65

It has been shown that CDC42Hs has a reduced affinity for the activated form of rat brain PAK65 and it was suggested that phosphorylation of PAK is a mechanism by which rac/CDC42Hs can be released from PAK once activated (Manser *et al.*, 1994). We have tested this model for hPAK65. Fully phosphorylated and unphosphorylated hPAK65, as judged by a mobility shift assay and kinase activity, bound equally well to activated CDC42Hs (Figure 5D). Phosphorylation of hPAK65 most likely serves to activate the kinase of hPAK65, but does not alter its affinity for rac/CDC42Hs.

Phosphorylated hPAK is an active kinase

Phosphorylation of hPAK65 induced by rac1 or CDC42Hs stimulates its kinase activity towards the substrate myelin basic protein (MBP) (Figure 6A). Both rac and CDC42Hs were able to stimulate an active hPAK65 kinase in a time-dependent manner. Maximal MBP phosphorylation was obtained within 20 min at 30°C (Figure 6B)

Activated hPAK65 does not require rac1 or CDC42Hs to sustain its kinase activity

The above experiments clearly demonstrate the requirement for rac1/CDC42Hs in the activation of hPAK65 autophosphorylation. However, it was not clear whether rac1 and CDC42Hs are required for hPAK65 kinase activity. To address this issue, we have designed the following experiment. hPAK65 autophosphorylation was first induced by CDC42Hs in the presence of non-radiolabeled ATP, then the hPAK65 and CDC42Hs complex was disrupted by exhaustive washes with lysis buffer. The autophosphorylated hPAK65 free of CDC42Hs (confirmed by Western blot) was subjected to a second kinase reaction containing [γ-^{32}P]ATP and MBP. Autophosphorylated hPAK65 free of CDC42Hs was constitutively active, as judged by MBP phosphorylation (Figure 6C). The control lane contained hPAK65 treated in exactly the same way except that ATP was not included in the first kinase reaction. Low levels of hPAK65 autophosphorylation were detected in the control hPAK65, most likely due to residual levels of unwashed CDC42Hs/PAK complexes. These data suggest that rac and CDC42Hs play an

important role in the activation of hPAK65 by stimulating its autophosphorylation, but not in regulation of the kinase activity.

The effect of hPAK65 on intrinsic and stimulated GTPase activity

Since the p65 protein was initially detected by the overlay assay as a potential effector and GTPase inhibitor for rac/CDC42Hs, we have examined the effect of hPAK65 on GTPase activity in solution. hPAK65 does exhibit a marginal effect on CDC42Hs intrinsic GTPase activity (data not shown). Increasing the amount of hPAK65 up to 1000-fold over CDC42Hs inhibited the intrinsic GTPase activity, but only by 10–15%. Interestingly, activated hPAK65 had an identical effect, suggesting that phosphorylation has no regulatory effect on rac/CDC42Hs intrinsic GTPase activity. In contrast, when the catalytic domain of p190 GAP (Settleman *et al.*, 1992) was included in the assay, hPAK65 exerted a significant inhibition on GTP hydrolysis stimulated by p190 (data not shown). Increasing the concentration of hPAK65 5-fold greater than that of CDC42Hs resulted in blocking by up to 80% of GAP-stimulated GTP hydrolysis, suggesting that p190 and hPAK65 compete for interaction with CDC42Hs or at least that hPAK65 significantly affects the interaction of p190 and CDC42Hs. Both phosphorylated and unphosphorylated forms of hPAK65 behaved identically in this assay and an affinity of ~200 nM for the hPAK65/CDC42Hs interaction could be extrapolated.

Discussion

Currently only a few effector molecules for rho-like proteins are known, including the new rat brain kinase PAK65 (Manser *et al.*, 1994) and p67-*phox* of the NADPH oxidase (Diekmann *et al.*, 1994). By utilizing the overlay assay we detected three novel targets for rac/CDC42Hs in neutrophil cytosol. These proteins are abundant in cytosolic fractions of neutrophils and HL-60 cells, which were used as a source for purifying the protein. The amino acid sequence of a peptide derived from purified p65 revealed a 100% identity with the kinase domain of brain PAK65. A full-length cDNA clone obtained from a human placental library also displayed striking sequence similarity to the kinase domain of rat brain PAK65 and yeast STE20. Although both rat brain PAK65 and hPAK65 exhibit similar specificity for rac/CDC42Hs, the clones display only 70% sequence identity in the rac1/CDC42Hs binding domains. It is most likely that this sequence functions as a regulatory domain and may interact with different regulatory proteins. Whether or not these sequence differences represent functional divergence in different cell types will need to be addressed. Our data suggest that hPAK65 is an isoform and not the human homolog of rat brain PAK65, since a specific antibody developed against the N-terminus of rat brain PAK65 recognized PAK protein only in rat and human brains and did not cross-react with recombinant hPAK65 and p65. In contrast, the hPAK65 antibody most probably recognized many members of the PAK protein family in various tissues, including purified neutrophil p65. This is consistent with recent data (M.Hutchinson and M.Cobb, personal communication) which identified additional rat PAK65 clones. One of the clones, named PAK2, exhibits >90% identity with hPAK65. This most likely represents the rat homolog of hPAK65 and not the previously published rat brain clone, which shares only 72% identity with hPAK65.

We have also shown by Northen blot analysis that PAK mRNA is ubiquitously distributed among various tissues, with higher levels in cells of myeloid origin. We designed a probe to detect closely related PAK messages, derived from the most conserved region of hPAK and rat brain PAK. Using the overlay assay, a high level of rat PAK proteins was detected mainly in brain cells (Manser *et al.*, 1994), whereas we find higher expression of hPAK65 in neutrophils and HL-60 cells. It is most likely that the overlay assay is not sensitive enough to detect PAK in tissues with relatively low expression of the protein and hence it may not be a suitable method for determining the tissue distribution of PAK.

We generated a peptide antibody derived from the kinase domain of PAK65 to determine whether the other two effector proteins, p62 and p68, identified in neutrophil cytosol are related to PAK proteins. No cross-reactivity was observed, suggesting that these proteins are different effectors for rac/CDC42Hs. Further characterization is required to identify these proteins.

By expressing hPAK65 in Sf9 cells we were able to purify an ample amount of recombinant hPAK65 for use in characterizing the biochemical properties of hPAK65. Like neutrophil p65 and rat brain PAK65, recombinant hPAK interacted specifically with the activated forms of rac1/CDC42Hs and subsequently, if provided with ATP, became autophosphorylated. Our data suggest that rac/CDC42Hs mediates hPAK65 autophosphorylation, thereby generating an active kinase. The strict requirement for hPAK65 binding and activation by only the GTP-bound form of rac and CDC42Hs indicates that hPAK65 may serve as an effector protein for rac/CDC42Hs. In addition, it is most likely that the relative binding affinities of rac/CDC42Hs for hPAK65 are regulated by their nucleotide state and not by the phosphorylated state of PAK65. In contrast to the model suggested (Manser *et al.*, 1994), our data suggest that the phosphorylation state of hPAK65 is not involved in regulating rac/CDC42Hs binding. Phosphorylated and unphosphorylated hPAK65 exhibited comparable affinities for rac1 and CDC42Hs.

Rac1 and CDC42Hs share ~72% sequence identity and have distinct physiological roles. For instance, rac1 induces membrane ruffling (Ridley *et al.*, 1992) and interacts with p67-*phox* to activate NADPH oxidase (Diekmann *et al.* 1994), whereas CDC42Hs has no effect on NADPH oxidase activity or on the induction of membrane ruffles. In yeast, CDC42Hs is involved in bud formation (Johnson and Pringle, 1990). The relatively lower affinity of hPAK65 for rac1 compared with CDC42Hs may suggest that CDC42Hs is the physiological activator of hPAK65. It is likely that rho-like proteins have numerous effector domains, some of which may be shared among the various family members, whereas others may be unique to specific members.

The partial phosphopeptide maps we have generated from hPAK65 activated by either rac or CDC42Hs were identical and indicate that rac and CDC42Hs activate hPAK65 in the same fashion, namely by stimulation of autophosphorylation at the same sites.

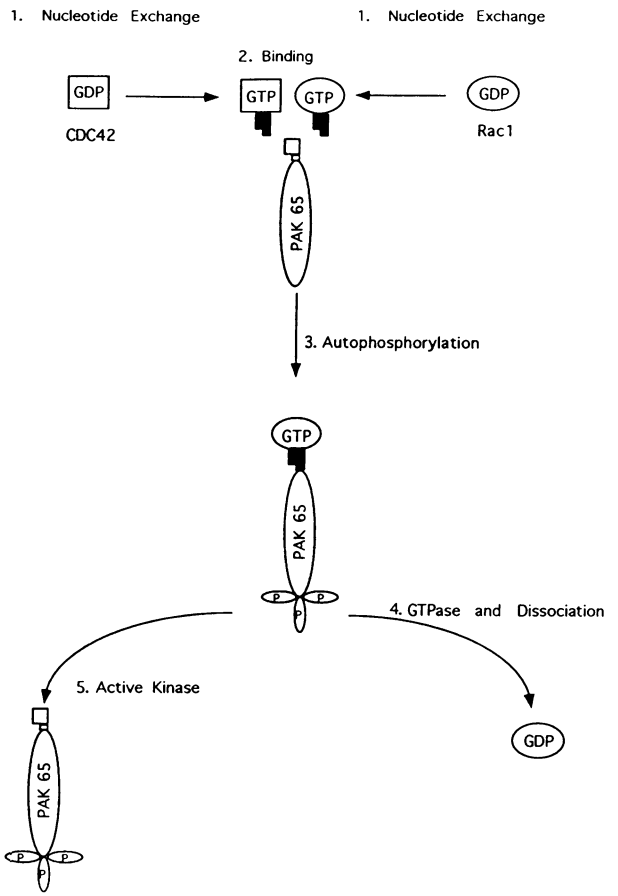

Fig. 7. A model of the role of rac1/CDC42Hs in the activation of hPAK65 kinase. (1) rac1 or CDC42Hs exchange factors are stimulated and release GDP and subsequently bind GTP. (2) The activated rac1 or CDC42Hs binds to hPAK65 (3) rac/CDC42Hs induces autophosphorylation of hPAK65. (4) The rac/CDC42Hs intrinsic GTPase hydrolyzes the GTP to the inactive GDP state. (5) The GDP-bound form of rac/CDC42Hs dissociates from the active autophosphorylated hPAK65 kinase.

It will be very important to determine which agonist will link rac1 or CDC42Hs stimulation with hPAK65 activation *in vivo*. Such studies will determine which nucleotide exchange factor for rho-like proteins is implicated in this pathway. For example, Dbl has been shown to have a nucleotide exchange activity on rho and CDC42Hs, but not on rac1 (Hart *et al.*,1991). Thus it is possible that activation of Dbl may lead to CDC42Hs-dependent hPAK65 stimulation.

The homology of hPAK65 and rat brain PAK65 to the kinase domain of yeast STE20 may provide a hint for the role of rac/CDC42Hs and PAK proteins in the MAP kinase cascade in mammalian cells (Avruch *et al.*, 1994). STE20 has been shown to be a target for the βγ subunits of the hetrotrimeric G protein in *Saccharomyces cerevisiae*, which link the pheremone response to a kinase cascade leading to trancription activation (Leberer *et al.*, 1992; Errede and Levin, 1993). Five protein kinases (STE20, STE11, STE7, FUS3 and KSS1) have been implicated between the G proteins and the transcription factor STE12 (Errede and Levin, 1993). STE7 has some homology to MAP kinase kinase and FUS3 and KSS1 are yeast homologs of MAP kinase (Errede *et al.*, 1993). In addition, genetic evidence demonstrates a functional association between a novel gene product STE5 and STE20 (Leberer *et al.*, 1993). Besides a limited homology to FAR1, STE5 has no known specific structural motif and most likely functions as an adaptor protein (Errede and Levin, 1993; Leberer *et al.*, 1993). The relatively high divergence between brain rat PAK65 and hPAK65 in their regulatory domains suggests that these proteins may be controlled by different molecules. Thus it is tempting to speculate that different mammalian STE5 homologs may serve as adaptor proteins to assemble distinct PAK-like kinases with downstream kinases such as STE7 and STE11.

In summary, our data are consistent with the model presented in Figure 7. Upon exchange of the nucleotide from GDP to the active GTP form, rac1/CDC42Hs interact with hPAK65 and subsequently induce hPAK65 autophosphorylation. The GTP is hydrolyzed to GDP by the intrinsic GTPase activity of rac1/CDC42Hs. The inactive GDP-bound rac1/CDC42Hs is released from the active hPAK65 kinase, which subsequently phosphorylates an as yet unidentified physiological substrate(s).

Materials and methods

The overlay assay

The method is a modified form of the one described by Manser *et al.* (1992). Briefly, 40 μl crude or partially purified fractions containing p65 (~80 μg protein) were applied to a 14% SDS−PAGE gel and blotted to a PVDF membrane. The membrane was stained for 30 s with Coomassie Blue stain to detect transferred proteins, destained for 2 min and incubated for 30 min with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 0.5 mM $MgCl_2$, 0.1% Triton X-100, 5 mM dithiothreitol (DTT). Either CDC42Hs, rac1 or rho (recombinant protein prepared in Sf9 cells) (30–50 μl, 3–5 μg protein) were diluted in 200 μl exchange buffer [25 mM 2-(*N*-morpholino)ethanesulfonic (MES), pH 6.5, 50 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 1 μl [γ-^{32}P]GTP (5 μCi; ICN) or [β-^{32}P]GDP (5 μCi; ICN)]. The proteins were incubated with the exchange buffer for 15 min at room temperature and were then mixed with 10 ml binding buffer containing 25 mM MES buffer, pH 6.5, 0.5 mM GTP, 5 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT. Immediately thereafter, the nucleotide-loaded protein was used to probe the filter. The mixture was incubated for 5–8 min and washed for 5 min with 25 mM MES buffer, pH 6.5, 5 mM $MgCl_2$, 0.05% Triton X-100. The membrane was dried and exposed to film for 2–3 h.

Protein purification

Cytosol was prepared from human neutrophils as previously described (Abo *et al.*, 1994). All purification steps were performed on columns connected to a FPLC system at 4°C and a flow rate of 1 ml/min and 1 ml fractions were collected. Neutrophil cytosol (10 ml, 10 mg/ml) was applied to a Mono Q column (HR5/5; Pharmacia LKB) equilibrated with buffer A [20 mM Tris−HCl, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg /ml pepstatin]. The proteins were eluted with a 30 ml gradient of from 0 to 0.5 M NaCl and the collected fractions were assayed for rac or CDC42Hs binding by the overlay assay. Fractions containing the p65 protein were pooled and subjected to ammonium sulfate precipitation. Ammonium sulfate grains were added to the mixture over a period of 15 min to achieve 40% saturation. The solution was stirred on ice for an additional 30 min and subsequently centrifuged at 100 000 *g* for 15 min in a TLX Beckman ultracentrifuge. The pellet was resuspended in buffer A to its original volume and the fractions were analyzed by the overlay assay. The supernatant obtained by 40% ammonium sulfate sedimentation, which contained the desired p65 protein, was further purified on a Phenyl Superose column (HR 5/5; Pharmacia LKB) equilibrated with 100 mM phosphate buffer, pH 7.2, 1 mM DTT, 5 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml pepstatin and 1.2 M ammonium sulfate. The bound proteins were eluted with a 30 ml gradient of from 1.2 to 0 M ammonium sulfate. In the case of the purification of a large amount of starting material (300–500 mg protein), the ammonium sulfate and the Phenyl Superose steps were the first purification procedures, followed by Mono Q fractionation. For this purpose we used a Hiload Phenyl Sepharose

16/10 column (Pharmacia LKB). Collected fractions were analyzed by the overlay assay and fractions containing the p65 protein were pooled and desalted into buffer A on a PD 10 column (Pharmacia LKB). The partially purified p65 was further purified on a Mono S column (HR5/5; Pharmacia LKB) equilibrated with buffer A. Proteins were eluted with a 30 ml gradient of from 0 to 0.5 M NaCl and the fractions were analyzed by the overlay assay. At this stage the p65 polypeptide could be easily identified by Coomassie Blue staining and the band was excised and used for amino acid analysis.

Peptide sequencing

The preparation was purified by SDS–PAGE, following staining with Coomassie Blue G-250, and the protein was excised. After washing, gel pieces were macerated and digested with *Achromobacter lyticus* endoproteinase Ly-C. Peptides were recovered by sequential washes and separated by tandem HPLC using 2.1 mm internal diameter anion exchange and reverse phase columns in series, following previously described procedures (Kawasaki and Suzuki, 1990). Fractions were collected and applied directly to an Applied Biosystem 477A pulsed liquid automated sequencer modified for fast cycle chemistry as described (Totty *et al.*, 1992).

Cloning the cDNA of p65

Based on the published protein sequence of rat brain PAK65 (Manser *et al.*, 1994) and amino acid sequence derived from purified p65 we designed the oligomers GM749, 5′-GGGGCCATCCAATAGGGGGTA-CCNACCATNG-3′, and GM752, 5′-ACCGGAGAATTCACCGGCAT-GCCTGAACAGTGG-3′, to amplify human cDNAs encoding PAK proteins. These oligomers were used to amplify specific PAK cDNAs from several commercially available human cDNA libraries. Because of the ease with which a specific product was obtained, we chose a human placenta library from Stratagene as a source for a cDNA clone. The gel-purified 962 bp PCR product was amplified in the presence of [^{32}P]dCTP and [^{32}P]dGTP, resulting in a radioactive product. This PCR product was used to screen ~100 000 recombinant plaques using stringent hybridization conditions. One positive clone was plaque-purified and auto-excised from λZap II according to the manufacturer's protocols. The sequence of the cDNA insert contained within the resulting Bluescript plasmid was determined using the dideoxynucleotide chain termination method.

Production of recombinant proteins in Sf9 cells

A Glu–Glu epitope tag was cloned onto the N-terminus of rac1, CDC42Hs and rho using the polymerase chain reaction (Grussenmeyer *et al.*, 1985). hPAK65 was myc tagged by ligating annealed oligomers into the *Xba*I site (417 bp). The tagged cDNAs were cloned into the baculovirus expression vector pAcC13. Snap frozen Sf9 pellets (1 g) expressing the desired proteins were Dounce homogenized in 10 ml buffer B (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 200 μM GDP, 1 mM Pefabloc, 10 μg/ml leupeptin, 10 μg/ml aprotinin). To remove the particulate fraction, the homogenate was centrifuged at 100 000 *g* for 15 min. The soluble fraction was applied to a 2 ml protein G–Sepharose column conjugated with either Glu–Glu or myc monoclonal antibodies. The column was washed with 10 ml buffer B lacking GDP and the protein was eluted with the same buffer containing either the myc peptide or 50 μg/ml of ED peptide (EYMPTD). Fractions were analyzed by SDS–PAGE, quantitated by the Bradford method, concentrated in a Centricon 10 (Amicon) to 1 mg/ml, aliquoted, snap frozen and stored at −70°C. A fresh aliquot of the protein was used for each assay.

Protein kinase assay

Recombinant hPAK65 (1–2 μg bound to protein G–Sepharose conjugated with monoclonal myc antibody) was washed once and incubated in 40 μl kinase buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$ with 1–2 μg of either rac1, rho or CDC42Hs, all previously loaded with GTP or GDP. The reaction was initiated by adding 10 μl kinase buffer containing 50 μM ATP and 5 μCi [γ-^{32}P]ATP and incubated for 20 min at 30°C.

The reaction was stopped by adding 10 μl 5× SDS–PAGE sample buffer and boiling for 5 min. Samples were applied to a 14% SDS–PAGE gel, which was stained with Coomassie Blue, destained, dried and exposed to film for 1–2 h. Phosphorylated bands were excised and the incorporated labeled phosphates were counted. In the case of MBP phosphorylation, 3 μg MBP (Sigma) were included in the kinase reaction.

Intrinsic and p190-stimulated CDC42Hs GTPase assay

Purified CDC42Hs (800 nM) was pre-bound to 80 nM [γ-^{32}P]GTP (6000 Ci/mmol) in the presence of 1 mM EDTA for 5 min at 25°C, followed by addition of 19 volumes GAP assay buffer (50 mM MES, pH 6, 100 mM NaCl, 5 mM $MgCl_2$) to yield 4 nM [γ-^{32}P]GTP–CDC42Hs. [γ-^{32}P]GTP–CDC42Hs (1 nM) was incubated with the indicated concentrations of hPAK65, which had been pre-incubated in the presence or absence of ATP. Reactions were carried out in GAP assay buffer containing BSA (0.2 mg/ml BSA, 50 mM MES, pH 6.5, 100 mM NaCl, 5 mM $MgCl_2$) in the presence or absence of 20 nM p190 catalytic fragment for 5–10 min at 25°C, followed by assay for phosphate release (Shacter, 1984). For p190-stimulated reactions, corresponding reactions in the absence of p190 were performed and the resulting hydrolysis subtracted to yield only p190-dependent activity.

Northern blots

To determine the tissue distribution of mRNAs encoding PAK, we used a radioactive PCR product containing the highly conserved kinase domain (bp 1009–1912) of human PAK cDNA to hybridize mRNA derived from 16 human tissues and eight cancer cell lines immobilized on Northen blots (Clontech). The hybridization conditions used were as suggested by the manufacturer for express hybridization, except for the temperature used (72°C) and time (overnight).

Antibody production and Western blots

Polyclonal antibodies were raised against recombinant hPAK65 in rabbits (Berekely Antibody Co.). Antibodies against the N-terminus of the rat brain protein (MSNNSLDVQDKPC) were a gift of S.Pelech and are now commercially available from Upstate Biotechnology Inc. (UBI). Human multiple tissue Western blots (75 μg protein/lane; Clontech Laboratories Inc.) were first probed with anti-N-terminus rat brain PAK65. Then the membrane was stripped and re-probed with hPAK65 antibody. Rat brain was homogenized in hypotonic buffer with protease inhibitors and the insoluble and particulate fractions were separated by centrifugation. Rat brain cytosol (75 μg), 0.2 μg recombinant hPAK65 and ~0.1 μg partially purified p65 were applied to each lane and analyzed with both antibodies.

Phosphoamino acid analysis

Recombinant hPAK65 (4 μg on 50 μl beads) was subjected to the kinase reaction as described above. The phosphorylated hPAK65 immobilized on Sepharose G beads was washed three times in PBS containing 1% Triton X-100 and then hydrolyzed in 50 μl 6 N HCl at 100°C for 2 h. The beads were removed by centrifugation and the supernatant was dried and dissolved in 10 μl electrophoresis buffer, pH 3.5 (10:100:1890 pyridine:acetic acid:water). Phosphoamino acids were resolved on a thin layer cellulose plate using the electrophoresis buffer essentially as described before (Cooper *et al.*, 1983). Standards were visualized by staining with 0.2% ninhydrin in acetone and ^{32}P-labeled residues were detected by autoradiography overnight.

Proteolytic digestion of hPAK65

To determine the phosphorylation pattern on hPAK65 induced by rac1 and CDC42Hs, hPAK was subjected to the kinase reaction containing either activated rac1 or CDC42Hs. To remove rac1 and CDC42Hs, hPAK65 immobilized on beads was washed three times with PBS containing 1% Triton X-100 and the beads were resuspended in 100 mM Tris–HCl, pH 6.8, 0.5% SDS, 10 mM DTT, 10% glycerol. The samples were boiled for 3 min and 10 μg of the indicated protease (all from Boehringer) were added. The proteins were digested overnight at room temperature and the samples were analyzed on 16% Tricine gels. The gels were stained with Coomassie Blue, destained, dried and exposed to film overnight.

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